Saliva from healthy men and women after exposure to three amorphous black raspberry confections: an analysis of salivary kinetics and polyphenol profile in oral cancer prevention

Thesis

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Abstract:

According to the American Cancer Society, an estimated 49,670 Americans will be diagnosed with oral cancer in the year 2016. Oropharyngeal cancer remains to be in the top ten most common cancer types and currently is the eighth most prevalent cancer in the United States. Oral cancer is regarded as a "lifestyle disease"—one that many dental professionals believe can be both caused and prevented by modifiable behaviors such as diet and oral hygiene practices. Previous studies have identified tobacco and alcohol use as risk factors for oral pharyngeal cancers; yet diets rich in fruits and vegetables have shown to reduce the risk of oral cancer. Among the many, black raspberry (BRB) has shown in previous rodent studies, anticancer activities toward oral cancer by inhibiting tumor development and downregulating genes involved in angiogenesis. Prolonging oral residence time using a confectionary matrix is one strategy to extend exposure of BRB compounds in the mouth, which arguably could enhance BRB absorption and reduce quantity of BRB needed for efficacy. However, some studies suggest that longer exposure in the oral cavity can enhance degradation of BRB compounds thereby diminishing their bioactivity. Therefore in this study, polyphenols in saliva were quantified and characterized after oral tumbling of three confectionary matrix having three different rates of release (slow, intermediate, and fast). We hypothesized that the confectionary matrix (modulated by the amorphous form) which provides the greatest oral residence time would cause greater quantities of BRB polyphenols to partition into the saliva and subsequently into the oral cavity and to the rest of the body. The following objectives were met: to determine the impact of oral residence time on the polyphenol profile of saliva in men and women after consumption

of three BRB confections and to evaluate the role of fruit and vegetable intake as well as oral hygiene practices on polyphenol profiles in saliva. In a four-week, randomized, phase I study, 30 men and 30 women, (non-smokers) evaluated three amorphous confectionary forms having three different release rates (prolonged, intermediate, rapid), each containing 1.25g freeze-dried BRB. HPLC with tandem mass spectroscopy was used to quantify and profile BRB compounds and metabolites from the confections and saliva. All confections were well accepted. Oral residence time was 74% longer and saliva volume was 42% greater with glassy than the other two forms. Fifteen polyphenols were identified in saliva. Saliva after hard candy consumption had significantly ($p \leq 0.05$) higher ellagitannin and methyl ellagic acid malonyl pentoside compared to the other two confections. Extending oral residence time enhanced salivary concentration of polyphenols, suggesting that confections with extended release of BRB compounds may be a good strategy for future oral cancer prevention trials.

1. Introduction

1.1 Oral Cancer and Modifiable Behaviors

Oral cancer is regarded as a "lifestyle disease"—one that many dental professionals believe can be both caused and prevented by modifiable behaviors such as diet and oral hygiene practices. Previous studies have identified tobacco and alcohol use as risk factors for oropharyngeal cancers[1]. Typically, oral cancers occur in the later decades of life. They can originate in any tissue of the mouth, and usually begin as a primary lesion or by metastasis from another area. They exist in different stages (initiated cells, pre-neoplastic, neoplastic, and malignant tumor) in the mouth, but it is not until the later stage that symptoms are noticeable. They are generally found as advance stages of cancer, and recurrence of cancer is frequent. Once oral cells are initiated by environmental stressors, such as tobacco smoke, these cells can progress over time into neoplastic cells. For this specific reason, progression to oral cancer potentially can be interrupted by dietary chemopreventative agents found in black raspberries. Moreover, previous studies suggest diets rich in fruits and vegetables have shown to reduce the risk of oral cancer[1]. Dietary behavior can have a large impact on oral health and prevention of oral disease. A diet poor in phytonutrients may suppress immunity and encourage development of severe oral infections[2]. Additionally, an unhealthy diet can lead to dental caries and enamel erosion[2]. A study in Italy showed that 20-25% of oral cavity cancers among healthy individuals were the result of low consumption of fruits and vegetables. The risk for the population rose to 85-95% when alcohol consumption and tobacco use were considered[1]. In the past, dental health has been centered on reparative techniques, with little practice of prevention[3]. As the importance

of dietary behavior becomes more apparent, dietary prevention strategies are emerging for promoting oral health.

1.2 Black Raspberries in Oral Cancer

Among the many fruits, black raspberries (BRB) are extremely rich in polyphenols and have anti-proliferation[4], anti-inflammation[5], and apoptotic activity[6], which are important in prevention of oral cancer. Specifically anthocyanins, an abundant flavonoid in black raspberries, are thought to decrease cancer risk[7]. Mallery and colleagues found that anthocyanins, specifically cyanidin 3-rutinoside, cyanidin 3-xylosylrtuinoside, cyanidin 3-glucoside, and cyanidin 3-sambubioside, are the polyphenolic compounds in BRB that are responsible for the chemopreventative effects[7]. By examining the oral tissues, saliva, and oral microflora involved in anthocyanin metabolism, they found that the enzymes of the oral mucosa were all capable of anthocyanin enteric recycling, a process that greatly increases anthocyanin contact time, and ultimately the chemopreventative effects of BRB by reintroducing them into circulation[7]. Additionally, they found that the inter-individual differences among participants in the target tissue absorption metabolic bioactivation, and local retention of the BRB constituents affected the chemopreventative responsiveness[7]. Ultimately, this data can help indicate which patients would respond to BRB chemopreventative treatment.

Further, Walle and colleagues report that quercetin inhibited proliferation of oral cancer cells with a minimum effective concentration of 5 μmol/L. This same study reports that this inhibition was dependent on the hydrolytic activity of quercetin in the oral cavity, and the extent of the hydrolysis was dependent on a variety of external

factors, such as residence time of food and the oral mucosa[8]. Earlier studies have reported that different chemical structures of polyphenols are prone to different rates of bacterial degradation, and thus their bioactivity differs both locally in the mouth and systemically[9]. Findings, however, have been inconsistent and clinical studies are limited. Oral microflora, salivary enzymes, and oral epithelium all seem to play a role in the synthesis of bioactive polyphenols[7]. The extents of their effects vary among individuals, however, and further research is necessary to determine the therapeutic potential of increased polyphenol retention[7].

1.3 BRB Anthocyanin Degradation

Cancer cells exist in multiple stages of growth; therefore a complex mixture of phytonutrients readily found in whole BRB can provide a multi-faceted, biological effect following their exposure with long-term toxicity being minimal. BRB contains an abundance of compounds called ellagitannins and anthocyanins. Anthocyanins are a family of compounds which are known as flavonoids. They are found in abundance in BRB and are attributed to providing its purple color. The predominant anthocyanin found in BRB is cyanidin, specifically cyanidin-3-O-β-D-glucoside and cyanidin-3-O-rutinoside, which subsequently break down into a variety of degradation products (Figure 1). Studies have demonstrated that anthocyanins have anti-inflammatory and protective activity, and that their bioactivity could largely be attributed to its degradation products[10]. This is because the parent anthocyanin structure is known to quickly degrade and become outnumbered by their intermediate products when anthocyanins are consumed[10].

In the Ferras et al (2014) study, participants were given a 500 mg oral bolus dose of a ^{13}C-enriched anthocyanin ($^{13}C_5$-C3G), and after 48 hours, researchers found a total of 35 ^{13}C-labelled analytes in the serum, urine, and fecal samples[10]. In the serum, specifically, 17 ^{13}C-labelled compounds were found, including 13 derivatives of protocatechuic acid (PCA) and 1 derivative of phloroglucinaldehyde (PGA). The parent $^{13}C_5$-C3G concentration was 141 nM, while the PCA and PGA concentrations were 146 nM and 582 nM, respectively with elimination half lives occurring 2-3 hours after the parent compound. In the urine, 31 ^{13}C-labelled compounds were found, including 19 derivatives of PCA and 2 derivatives of PGA. Again, the degradation products were excreted in much higher concentrations than the parent compound. Additionally, there were 28 ^{13}C-labelled compounds identified in the feces[10].

Anthocyanin metabolites remain in circulation at a much higher concentration, and for a longer period of time than the parent compound. Ferras et al (2014) found that the parent anthocyanin only made up 2% of the total metabolites in circulation, and had a half-life that was significantly shorter than its metabolites[10]. The major degradation products of the parent anthocyanins in black raspberries are PCA and PGA, which are further degraded into a wide range of metabolites[10].

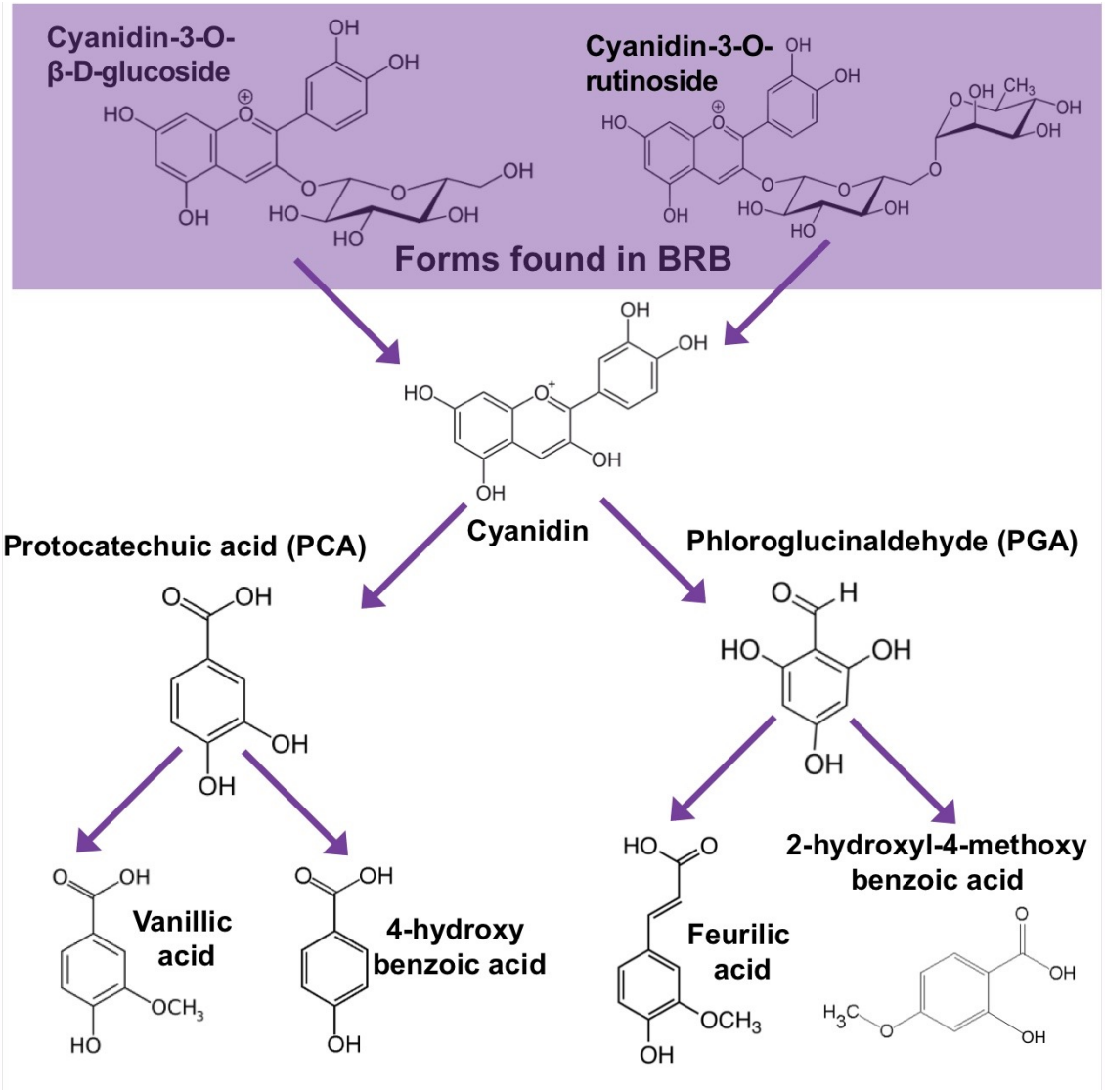


Figure 1. Black Raspberry anthocyanins, cyanidin (those found in BRB fruit, highlighted in purple), and possible breakdown products[6]

Kamonpatana and colleagues assessed the relationship of anthocyanin structure on metabolism and found variability in hydrolytic activity of flavonoids in the oral cavity. They analyzed five anthocyanin (ACN)-rich extracts containing 6 anthocyanidins in various mono-, di-, and tri-saccharide forms incubated in the saliva of 14 healthy individuals. Results indicated that all 21 ACN in the extracts were partially or completely

degraded, but the extent of degradation was dependent on the ACN structure. The degradation of disaccharide pelargonidin-3-rutinoside was more than 15% less than that of the monosaccharide pelargonidin-3-glucoside. Additionally, the degradation of the tri-saccharide, xylosylrutinoside was about 10% lower than degradation of the disaccharide rutinose cyanidin conjugate[9]. The pattern of degradation for each of the individual subjects varied, but at least 50% of the chokeberry ACN was degraded in the saliva by the majority of the participants, indicating that the greatest factor in the extent of degradation was the anthocyanin structure[9]. Moreover, when degradation of chokeberry ACN was examined on a single subject, they found degradation of ACN in saliva to increase in a time-dependent manner[9]. After a 60 minute incubation, approximately 50% of each of the four cyanidin glycosides was degraded[9]. From these results, Kamonpatana and colleagues concluded that, although the anthocyanin structure influenced degradation, the type of monosaccharide had little effect on the extent of ex vivo degradation[9].

Additionally, Kamonpatana and colleagues (2012) examined the degradation products of chokeberry cyanindin glycosides in saliva to determine whether the loss of chokeberry cyanidin glycosides in saliva was associated with the generation of cyanidinaglycone and its pheonolic products[9]. Typically, the microbial metabolites, protochatechuic acid or phloroglucinol aldehyde, account for up to 20% of degradation of cyanidin glycosides during incubation with intestinal microbiota in human and pig feces[9]. When incubated with intact saliva, however, neither PCA nor PGA was detected. This indicated that the disappearance of cyanidin glycosides and their aglycone was not coupled with increases in PCA or PGA, despite them both being cyanidin degradation

products[9]. These findings, along with the presence of chalcone glucosides of cyanidin, suggest an alternative degradation pathway of anthocyanin in the oral cavity different than that of degradation in the large intestine[9].

2. Problem Statement

Mixed results reported from previous investigations on polyphenol bioavailability may be due to the individual differences in salivary kinetics and salivary variations interacting with BRB compounds in the oral cavity. One hypothesis suggests extending the exposure of BRB compounds in the mouth will enhance their absorption and improve their bioactivity. Yet another hypothesis indicates that the prolongation of BRB residing in the oral cavity will enhance their degradation by oral bacteria thereby diminishing their bioactivity. Profiling polyphenols in saliva following the release of the confections at different rates will help elucidate whether confections with the greatest residence time in the oral cavity will result in the greatest quantity of polyphenols to partition into the saliva. Ultimately, findings will add to our knowledge in providing nutritional recommendation to promote oral health towards prevention of oral cancer.

Currently, there is limited information on the impact of saliva on polyphenol profile of BRB. Saliva contains a variety of enzymes that work to hydrolyze foods within the oral cavity and there is great inter-individual difference in the quantity and profile of these enzymes. Recent studies have analyzed salivary amylase, the enzyme responsible for starch hydrolysis. The study determined that individuals with high-starch diets had greater levels of amylase protein, which in turn increased the ability for starch digestion in the mouth. Thus, greater exposure to starch resulted in a greater efficiency

with which starchy foods were digested[11]. In addition to salivary enzymes, bacteria in the oral cavity are also believed to be responsible for hydrolysis and degradation of polyphenols, and ultimately the delivery of bioactive compounds[8]. These differences in host salivary enzymes and oral microbiome in combination contribute to individual differences in BRB metabolism and thereby dictate individual's disease outcome. The overall goal of this study is to clarify mechanistically what factors influence BRB metabolism in the saliva, and the role that these BRB metabolites have in oral health.

Therefore to investigate the impact of oral residence time on BRB polyphenol profiles in saliva, three amorphous forms (glassy: hard candy, viscous: pectin-gummy, elastic: starch-gummy) were designed to mediate the length of BRB exposure in the oral cavity. We hypothesized that saliva collected after exposure with the amorphous confectionary form having the longest oral residence time will have the greatest concentration of anthocyanidins compared to the other two amorphous forms. This study met the following objectives:

Objectives: A randomized crossover evaluation involving three amorphous confectionary forms (hard candy, pectin, and starch) having three different rates of dissolution were evaluated in 60 men and women where the following two objectives were met:

1. **To determine the impact of oral residence time on the polyphenol profile of saliva in men and women.**
2. **To evaluate the role of fruit and vegetable intake as well as oral hygiene practices on polyphenol profiles in saliva.**

3. Materials and Methods:

3.1 Study Design

Nested within a sensory evaluation of the three amorphous confections was an evaluation of confection durability. Participants arrived at the OSU Clinical Research Center after an 8 hour fast and a 1 hour abstention of water prior to testing. Men (n=30) and women (n=30) were asked to evaluate the duration of three different amorphous confections by recording the time needed to completely eliminate the confections from their mouths. Participants were asked to avoid chewing the confection and swallowing their saliva during the evaluation. Each confection contained 1.25 grams of lyophilized BRB. Subjects were to avoid eating and drinking one hour prior to their visit as well as avoid brushing their teeth prior to their visit. Confection presentation order for each participant was assigned in randomized counterbalance manner for 70 subjects, Figure 2. Participants were asked to tumble the BRB confection in their mouths and expectorate all their saliva into collection tubes. Saliva was collected from each individual after each confection sample. After each confection subjects were instructed to cleanse their palate using a whole water cracker (Carr's®, United Biscuits Ltd, UK) and rinse a minimum of three times with water. Subjects were instructed to repeat this procedure before each sample.

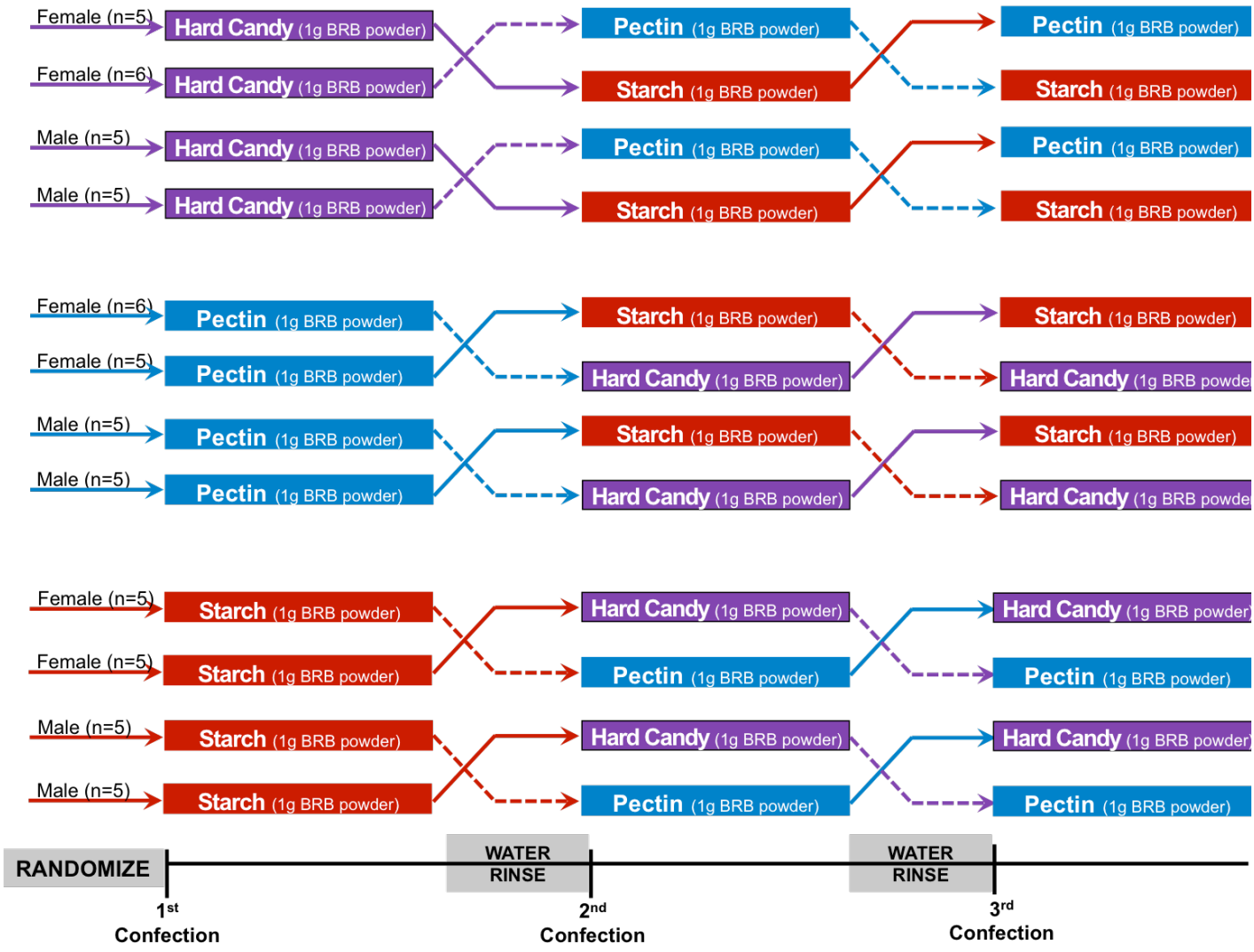


Figure 2. Study design of saliva collection from three amorphous confections presented in a serial monadic manner

3.2 Study Cohort

For this study, healthy, nonsmoking adults (30 men and 30 women) between the ages of 18 and 65 were recruited and enrolled. Subjects included on this study had a body mass index (BMI) between 18 and 35kg/m^2. They were non-smoker, which was defined as adults who had never smoked or who had not had a cigarette in the past ten years. Enrolled participants had to agree to consume a standardized vitamin/mineral supplement and avoid other nutrition and alternative supplements for the duration of the

study, as well as agree to follow a berry-restricted diet and to document any accidental consumption of restricted foods each day of the study. Participants had to abstain from mouthwashes during the entire 4 weeks of study. Subjects excluded from the study had active or a history of metabolic or digestive disorders, as well as altered immunity. Those participants with a known allergy or food intolerance to ingredients in study products (black raspberries other berries, wheat, or corn, or pectin) were prohibited from participation. Those who had a history of oral cancer or had any active oral lesion or oral maladies were excluded from the study, as well as a strong gag reflex or problems swallowing that prohibit buccal brushing of the oral cavity were excluded from participation. Those who were heavy alcohol consumers (defined as an average consumption of greater than 2 drinks/ day) or had been on an antibiotic regime lasting for one week in the last 6 months were discouraged from participation.

3.3 Study Agents

3.3.1 Confection Description

Three highly acceptable confection candidates (**Table 1**) with differing release rates (fast, intermediate, and slow) were selected as each arm of study treatment blocks. The glassy black raspberry (BBR) construct was a ~2 gram hard candy (6 calories/ piece), as was the pectin and starch BRB confection (5 and 6 calories/piece, respectively). Each study agent contained 1.25 gm BRB powder per piece.

3.3.2 Confection Preparation

Confections were prepared using a single lot of freeze-dried black raspberry powder. Black raspberry powder was procured from Dale Stokes Farms (Wilmington, OH) and freeze-dried by Van Duren Farms (Momence, IL). Confections were prepared as detailed by Gu et al (2015)[12]. Freshly prepared confections were individually packaged into 0.5 ounce soufflé cups, which were affixed with airtight lids and stored in amber bags at room temperature for no more than 48 hours prior to their administration.

Table 1. Finished Confection Formulation[12]

Composition (g)	Hard Candy	Pectin confection	Starch confection
BRB powder	1.25	1.25	1.25
Sugar	3.15	2.18	0.63
Corn syrup	1.85	0.75	2.28
Water	-	1.85	1.70
Starch/pectin	-	0.09 (Pectin)	0.39 (Starch)
50% (w/w) Citric acid	-	0.13	-
Total	6..25	6.25	6.25

3.4 Determination of Oral Residence Time and Saliva Collection (Appendix B)

Subjects were fasted for 8 hours and abstained from drinking water one hour prior to their visit as well as avoid brushing their teeth prior to their visit. Assignments to the order of confection presentation were done in a randomized counterbalance manner for 70 subjects (see above representative study design). Subjects were instructed in how to use a timer to time their oral residence time. They were instructed to tumble the confection around their mouths. They were prohibited from chewing the confection or swallowing their saliva during the saliva collection. Subjects were instructed to expectorate into a 50 mL polypropylene centrifuge tube (Corning, Corning, NY) all the

saliva that accumulated in their mouths while keeping the confection in their mouth. End time was when confection material was completely absent in their mouths. Participants were asked at the end of the study to make sure all saliva was expectorated into the tubes provided. The total volume of saliva was recorded and a 5 mL aliquot was stored at -80°C for future analyses.

3.5 Saliva Extraction and Analysis

3.5.1 Chemicals

Formic acid (Acros Organic, >96% purity), acetonitrile (HPLC grade), and water (HPLC grade) were purchased from Fisher Scientific.

3.5.2 Extraction

Frozen saliva samples (5mL) collected from each participant for each of the three confection samples were thawed using an ice bath. BRB polyphenols were extracted from 0.5 mL of saliva using 1 mL acidified (5% formic acid) acetonitrile (v/v%). The samples were lightly probe sonicated (Branson sonicator 150E - Fisher Scientific, NJ) for 15 seconds on continuous mode with amplitude set at 35. Following the probe sonicator, samples were kept on ice and were centrifuged (Centrifuge 5424 – Eppendorf, Hamburg, Germany) at max speed (21,130 x g) for 5 minutes. The supernatant was collected into a glass vial using a glass Pasteur pipet. The remaining pellet was resuspended with 1 mL of 66% acidified (5% formic acid) acetonitrile (aqueous, v/v%). Again, the sample was probe sonicated on the same settings, kept on ice, and centrifuged for another 5 minutes at max speed. The supernatant was again

collected and pooled with the first supernatant transfer. Saliva sample extracts were dried under a SpeedVac (SpeedVac Concentrator - Thermo Scientific Savant SPD131DDA, Waltham, Massachusetts) equipped with a refrigerator vapor trap (Savant RVT5105, Waltham, Massachusetts) and RV rotary vane pump (Edwards, Crawley, UK) for 4-5 hours. The SpeedVac was set to preheat temperature of 45°C for 30 minutes, followed by no heat during the remainder of the drying process. The vacuum was set with a ramp level of 5 (maximum setting). Dried samples were immediately stored at -80°C until HPLC analysis. A total of 192 saliva samples were extracted.

3.5.3 Analysis

The prepared extracts which were stored at -80°C were brought to room temperature and resolubilized using 80% methanol. Saliva extracts were analyzed using HPLC with tandem mass spectroscopy by Phytochemical Analytics Shared Resource using published methods for anthocyanins[8]. Extracts were analyzed using HPLC photodiode array (PDA) with tandem MS/MS and relative abundance was reported.

3.6 Demographic and Dietary Data Collection

3.6.1 Health and Lifestyle Questionnaire (Appendix C)

Health and Lifestyle questionnaire was used to collect demographic data and to assess if any participant needed to be excluded from this due to any pre-existing medical conditions, food allergies, or smoking/drinking behavior. This questionnaire was administered prior to their enrollment.

3.6.2 Three-Day Diet Records (Appendix D)

Self-reported 3-day diet records were collected on alternating days prior to their confection saliva collection. Discrepancies in the diet records were clarified by a short interview with dietitian. All data was collected and analyzed using NDSR (Nutrition Data System for Research) software (Minneapolis, Minnesota)

3.7 Statistical Analysis

Statistical evaluation was performed using SPSS software Version 24 (IBM Inc., Chicago, Il, USA) and *p-values* ≤ 0.05 were considered to be significant. Mean ± standard error mean was used to report saliva volume, oral residence time, relative abundance of BRB compounds in saliva, and demographic as well as dietary parameters unless otherwise specified. A one way NOVA (analysis of variance) was used to analyze overall differences among the three treatments. When significant differences were found, Tukey's posthoc test was used. Independent t-test was used to compare gender differences from 3-day diet records. Pearson correlation was conducted to find relationships between fruit and vegetable intake and healthy oral hygiene practice (brushing frequency and flossing).

4.Results/Discussion:

4.1 Study participants

Once IRB approval (IRB#2013C0056) was obtained, healthy, nonsmoking men and women were recruited (Appendix A). A total of 34 men and 34 women were enrolled with 30 men and 30 women completing all study activities. Six subjects were excluded

from analysis because at the end of study admitted that they were social smokers and two subjects dropped out due to undisclosed reasons. Demographic data of study cohort is stratified by gender, **Table 2**. No significant differences due to age or BMI were observed between men and women for this study cohort. Women had a slightly higher BMI than men.

Table 2. Demographic Oral Hygiene Practice

Age (mean ± SD)	
Men n=34	31±12 years old
Women n=34	33±11 years old
BMI (mean ± SD)	
Men n=34	26.4 ± 10.8 kg/m^2
Women n=34	28.6 ± 6.2 kg/m^2
Twice daily teeth brushing	
Men n=34	59% (20/34)
Women n=34	76% (26/34)
Daily flossing	69% (46/68)
History of dental surgery	13% (9/67)
Regular alcohol consumers (average 3.25 servings/ week)	69% (46/67)
Never smokers	89% (60/67)
Past smokers (0.5 pack/ 5.5 years)	11% (7/67)

4.2 Oral Residence Time and Saliva Volume

Oral residence of the three confectionary forms exhibited differences in oral duration. Hard candy demonstrated the greatest duration in the oral cavity as well as resulted in the greatest volume of saliva produced during saliva collection, Figure 3.

Previous studies have indicated that varying delivery matrices of BRB confections could lead to different release rates of phytochemicals from these confections. Gu et al (2015) examined three amorphous confectionaries: glassy (hard

candy) and two viscoelastic (pectin- and starch-based) confections[12]. They performed an *in vitro* dissolution study using artificial saliva to determine release rates of the confections. Hard candy had the fastest release rate of 75 minutes with 93.5% of total release. Pectin demonstrated an intermediate release rate of 180 minutes with 94.5% of total release, the highest release of total phenolics. And, starch exhibited the slowest release rate of 540 minutes with 78.7% of total release. They attributed these differences to gelling polymer addition and structure differences[12].

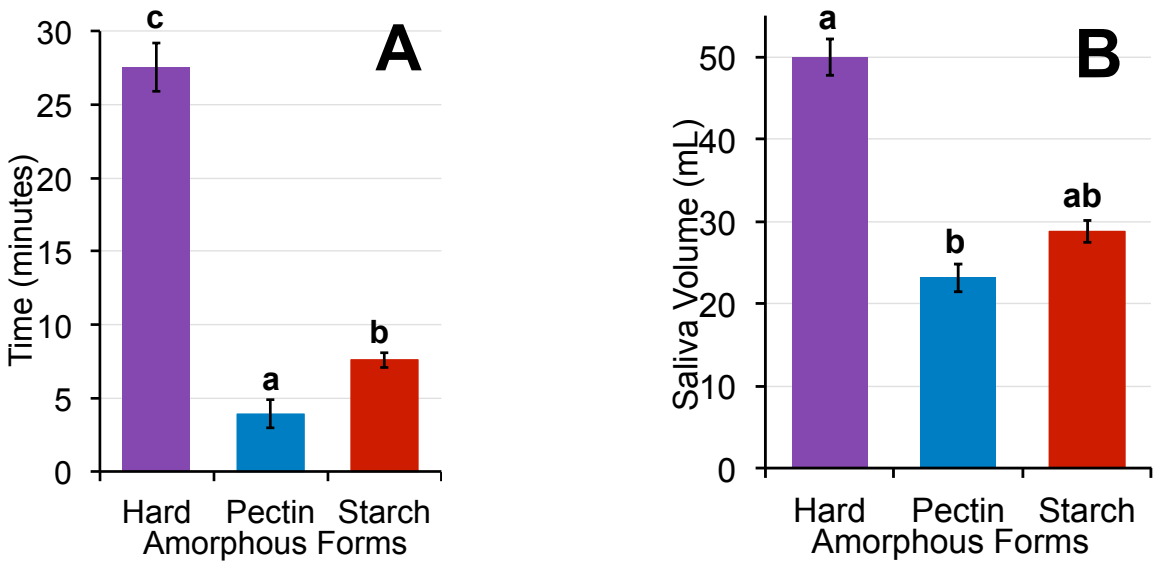


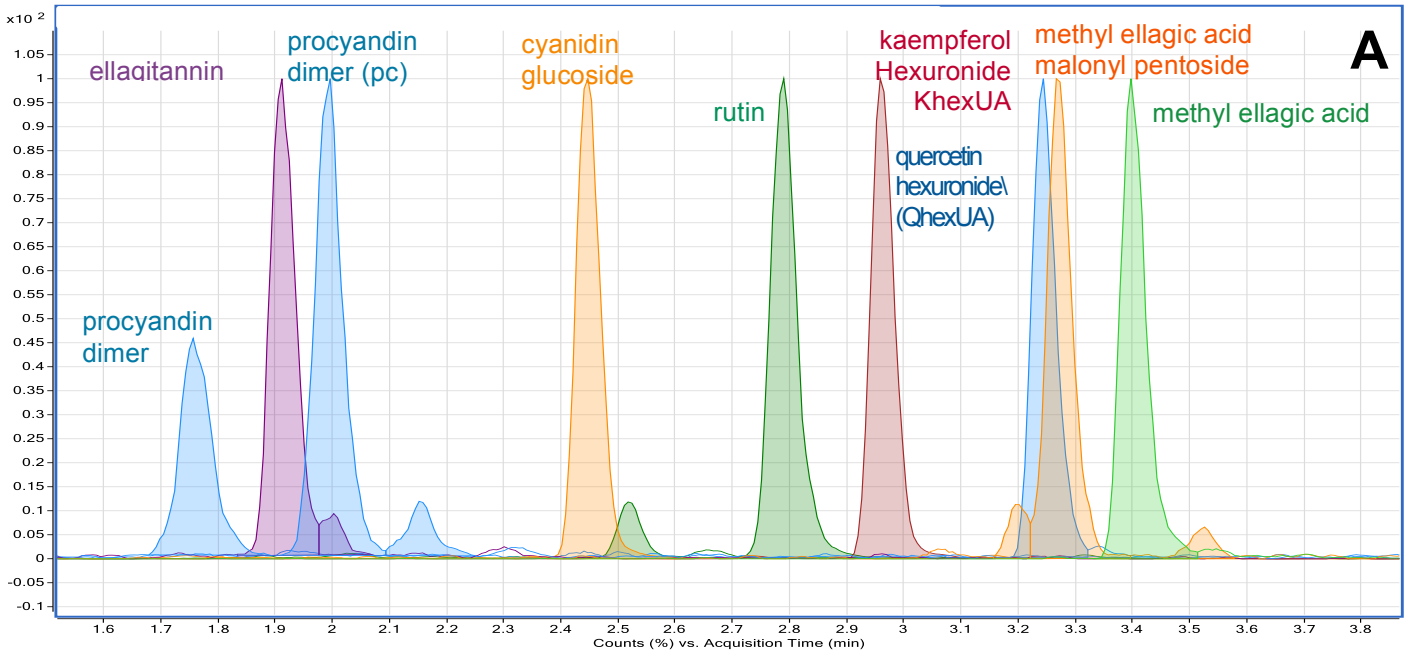
Figure 3. Oral residence time (durability) measured in minutes (A) and quantity of saliva produced during each amorphous confection evaluation (B). Confection duration was measured in a randomized crossover design to asses confection durability. Saliva collected during confection tumbling. Letters denote differences using ANOVA ($p \leq 0.05$) with Tukey's posthoc test

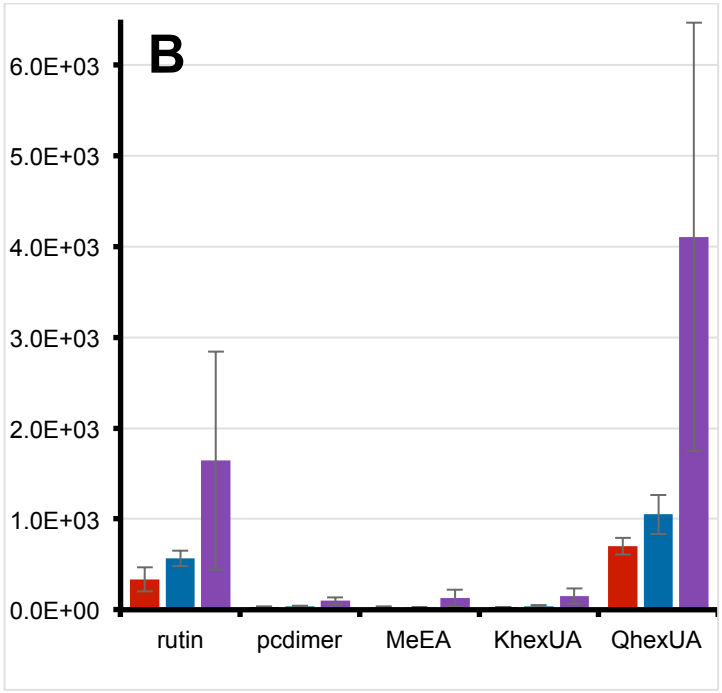
4.3 Polyphenol profile in saliva after confections

Chemical analysis of saliva collected during confection tumbling in the oral cavity discovered 15 metabolites of anthocyanin and ellagitannins, Figure 4A. Saliva from confectionary forms were combined from three individuals, hard candy produced

significantly greater amounts of ellagitannin and methyl ellagic acid malonyl pentoside in the saliva, Figure 4B and C. Past studies have found evidence of a positive relationship between time and degradation of anthocyanins. Kamonpatana et al (2012) found that when examining the extent of chokeberry anthocyanin degradation, degradation of the cyanidin glycosides increased with time[9]. Additionally, in a follow up study, Kamonpatana et al (2014) found that they could not detect levels of PCA, PGA, or other anthocyanin breakdown products in buccal cell extracts after a five-minute retention of chokeberries[13]. They suggest that increased oral residence time of anthocyanins may be necessary to produce the bioactive anthocyanin metabolites[13].

The results of this study report similar findings, indicating that the hard candy confection resulted in the greatest duration exposure in the saliva that we evaluated. The hard candy also produced a significantly greater amount of anthocyanin breakdown products, which are believed to be responsible for anthocyanin bioactivity[10]. This suggests that longer oral residence time results in a higher quantity of black raspberry compounds released in the oral cavity, and could potentially be a good strategy for future long-term oral cancer prevention trials.





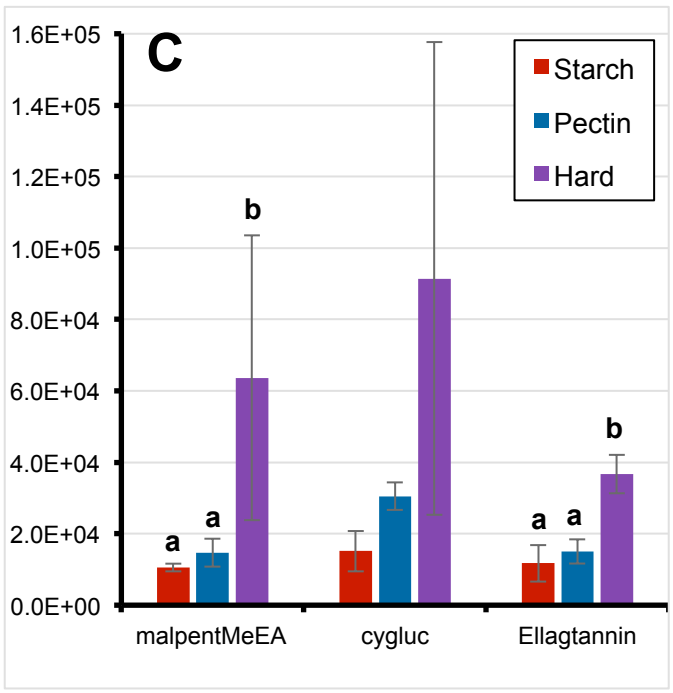


Figure 4. Representative HPLC-MS/MS analysis (triple quadrupole-Agilent) of saliva with BRB amorphous confection (A). Relative abundance of BRB compounds corrected to saliva volume among the three amorphous confection forms (n=9), (B and C). Letters denote mean separation where significant differences ($p \leq 0.05$) were found using ANOVA analysis with Tukey's posthoc test among the amorphous forms within a single dose.

4.4 Inter-individual differences in oral residence time of three amorphous confections. Three individuals were selected based on the length of time to tumble the confections and that they all had a sequence of confections starting with pectin, Figure 5. The first individual had a fast time (shortest oral residence, Figure 5A), the second intermediate (Figure 5B), and the third had a slow time (longest oral residence, Figure 5C). These individuals exhibited differences in the relative quantity of metabolites. The rapid tumbler shows really different and high relative abundance of chemical compounds, while the slower tumbler who allowed the confection to sit in his/her mouth for longer had lower levels of chemical compounds in their saliva. Essentially, the fast tumbler had the highest level of compounds in the saliva from the hard candy compared to the slow tumbler. Thus, from a confection perspective, longer residence time improved absorption, but too long of a residence time resulted in lower levels of compounds in saliva because too long of a duration can lead to degradation of the compounds.

The inter-individual differences in the relative quantity of metabolites among and within each of the three participants, supports the idea that further investigation is needed to understand why individual differences in metabolites occur and warrant further studies, which can lead to future understanding of opportunities towards personalized medicine. Mallery et al. (2011) noted these interpatient differences in anthocyanin bioactivation and metabolism, and that they would affect treatment effectiveness among patients[7]. By examining specific individuals in this study, we were able to take note of inter-individual differences in hopes of eventually creating personalized treatment plans.

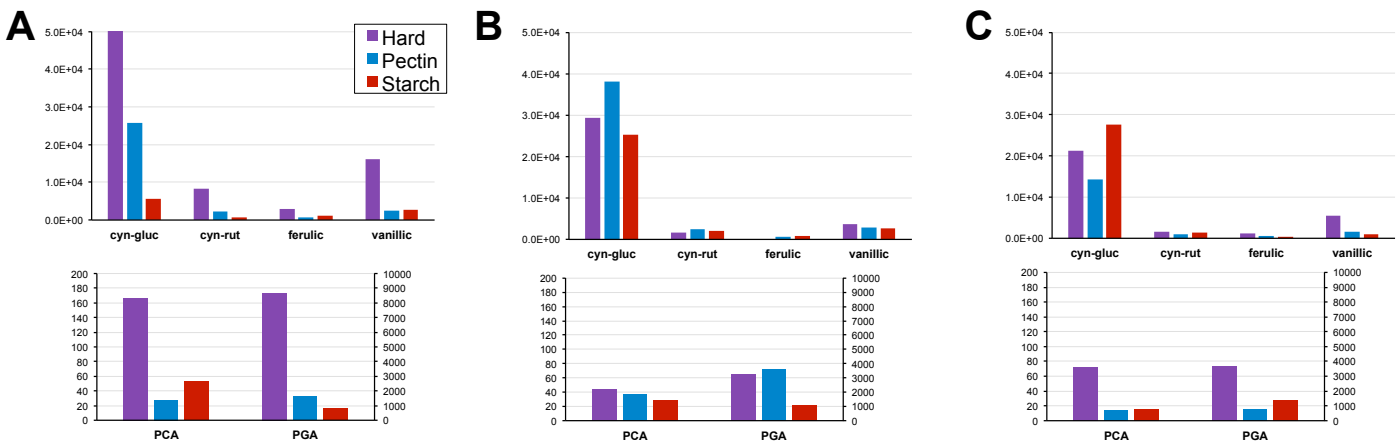


Figure 5. Individual polyphenol profiles based on relative abundance from saliva of three study participants having fast (A), intermediate (B), and slow (C) confection tumblers. Fast tumbler finished confection in 3 to 18 minutes. Intermediate finished between 5 to 27 minutes. Slow finished between 6 to 59 minutes. Cyn: cyaniding; gluc: glucoside; rut: rutinoside; PCA: Protocatechuic acid; PGA: Phloroglucinaldehyde.

4.5 Dietary Demographics

Health and Lifestyle Questionnaire as well as a 3-day diet record, were completed prior to the start of their intervention. Oral hygiene practices and dietary intake of fruit and vegetables were evaluated against BRB polyphenol metabolite phenotypes. Data from the 3-day diet records was entered into NDSR (Nutrition Data System for Research) software (University of Minnesota). Statistically significant differences among high and low fruit and vegetable consumers as well as those with good (brushing frequency, flossing, and mouth wash use) and bad oral hygiene practices was compared, Table 3.

Table 3. Three-Day Diet Records

Nutrient Parameters	Men n=30	Women n=32	p-values
Total Energy	1975 ± 127 kcal	1975 ± 127 kcal	0.0381*
Total Fat	80.1 ± 7.3 g	69.3 ± 6.9 g	0.0586
Cholesterol	322.8 ± 42.4 mg	217.9 ± 29.3 mg	0.0004*
Total Carbohydrates	217.8 ± 14.0 g	213.4 ± 18.6 g	0.7440
Total Dietary Fiber	18.9 ± 1.8 g	21.1 ± 2.4 g	0.1906
Total Protein	93.8 ± 7.2 g	74.7 ± 6.3 g	0.0005*
Total Fruit Intake (servings/day)	1.01 ± 0.19 g	1.89 ± 0.34 g	0.0441*
Total Vegetable Intake (servings/day)	3.65 ± 0.43 g	4.00 ± 0.37 g	0.5520

*Statistical differences using a independent t-test

Total energy, cholesterol, total protein, and total fruit intake were statistically different, which is expected since we are comparing men and women who have known differences in nutrient requirements. Fruit intake in women was significantly higher compared to men. However, we found no strong correlation (r=0.426) between fruit and vegetable intake and healthy oral hygiene practices. Previous studies have reported findings that suggest a correlation between fruit and vegetable intake and oral hygiene practices may exist. One study examined fruit and vegetable intake in edentulous patients with impaired chewing function. They found fruit and vegetable intake to be significantly lower in the edentulous participants compared to those with teeth[3]. The idea is that those that lack an adequate ability to chew are less likely to choose fruits and vegetables that require more chewing. On the other hand, those with their natural teeth still intact are more likely to choose fruits and vegetables because they are able to chew them[3]. Further correlations using NDSR data will be conducted to see relationships between polyphenolic profile and dietary patterns.

5. Conclusion

In conclusion, the hard candy confection provided the greatest duration exposure from the saliva that we evaluated. Additionally, the hard candy produced the greatest amount of saliva, as well as significantly greater amounts of ellagitannin and methyl ellagic acid malonyl pentoside released in the mouth. This suggests that extending oral residence time enhanced salivary concentration of polyphenols, which suggests that confections with extended release of BRB compounds may be a good strategy for future prevention trials for oral cancer.

We found substantial inter-individual differences observed in the rate of confection consumption, quantity of saliva produce, and polyphenolic profile. Each individual has a very different oral microbiome, and understanding these differences can help to create a personalized medicine approach with different treatments that work for different people.

In regards to the 3-day diet records, we found no correlation between fruit and vegetable consumption and dental hygiene practices. It is part of our secondary analysis, however, to continue investigating other correlations in our NDSR data from our additional samples waiting to be analyzed.

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Appendix A – IRB approval letter

Cancer Institutional Review Board

Office of Responsible Research Practices
300 Research Foundation
1960 Kenny Road
Columbus, OH 43210-1063
Phone (614) 688-8457
Fax (614) 688-0366
www.orrp.osu.edu

July 3, 2013

Protocol Number:	2013C0056
Protocol Title:	PHYTOCHEMICAL RELEASE RATE FROM BLACK RASPBERRY CONFECTIONS ALTERS GENE EXPRESSION AND CHAMICAL PROFILES RELEVANT TO INHIBITION OF ORAL CARCINOGENESIS, Yael Vodovotz, Steven Clinton, Steven Schwartz, Christopher Weghorst, Food Science & Technology
Type of Review:	Initial Review
IRB Staff Contact:	Ryan Liersemann 614-292-0243 Liersemann.1@osu.edu

Dear Dr. Vodovotz,

The Cancer IRB **APPROVED** the above referenced research.

Date of IRB Approval:	**July 2, 2013**
Date of IRB Approval Expiration:	**July 2, 2014**

If applicable, informed consent (and HIPAA research authorization) must be obtained from subjects or their legally authorized representatives and documented prior to research involvement. The IRB-approved consent form and process must be used. Changes in the research (e.g., recruitment procedures, advertisements, enrollment numbers, etc.) or informed consent process must be approved by the IRB before they are implemented (except where necessary to eliminate apparent immediate hazards to subjects).

This approval is valid for **one year** from the date of IRB review when approval is granted or modifications are required. The approval will no longer be in effect on the date listed above as the IRB expiration date. A Continuing Review application must be approved within this interval to avoid expiration of IRB approval and cessation of all research activities. A final report must be provided to the IRB and all records relating to the research (including signed consent forms) must be retained and available for audit for at least 3 years after the research has ended.

It is the responsibility of all investigators and research staff to promptly report to the IRB any serious, unexpected and related adverse events and potential unanticipated problems involving risks to subjects or others.

This approval is issued under The Ohio State University's OHRP Federalwide Assurance #00006378.

All forms and procedures can be found on the ORRP website – www.orrp.osu.edu. Please feel free to contact the IRB staff contact listed above with any questions or concerns.

W.E. Carson III

William Carson, M.D., Chair
Cancer Institutional Review Board

hs-017-04 Approval New CR
Version 01/13/09

Appendix B – Oral Residence Time

Instructions for Operation of Stopwatch

Please make sure BEFORE you began to sample your first candy sample that the timer is displaying 0:00. Please alert the study coordinator if this is not true.

1. Press the START/STOP button (blue arrow) at the top right of the timer after you have placed the candy in your mouth BUT before you begin to tumble the product. **PLEASE DO NOT PRESS ANY OTHER BUTTONS.**

2. Record only the numbers that are displayed on the timer, as depicted in the yellow box.

3. After the candy sample has completely dissolved in your mouth, make sure to stop the timer by pressing the same START/STOP button at the top right of the timer again. Record both the start and stop times for each product tasted.

4. For the next candy sample, record the start time and then press the same START/STOP button at the top right of the timer after you have placed your next sample in your mouth. Repeat steps 3 and 4 until you have evaluated all seven 7 candy samples.

Subject ID:_____

Black Raspberry Confections

CONFECTION # **123**

Please cleanse your mouth with the water cracker and rinse your mouth with water three times. Read all the instructions below before you begin

Place the entire sample in your mouth and make sure to start the timer provided.

Once the candy is in your mouth, start to vigorously tumble the sample in your mouth until the sample has completely dissolved. PLEASE AVOID CHEWING THE SAMPLE. You can swallow the saliva that accumulates. Stop the timer when you feel that there is no more candy left in your mouth.

SAMPLE: **123**

START TIME: _____ **END TIME: _____**

Did all of the candy dissolve in your mouth ? ☐ Yes ☐No (Go next question)

Which of the following was done to the remaining candy? ☐ Expectorated

☐Swallowed

Subject ID:_____

Durability Test

Black Raspberry Confections

CONFECTION # **456**

Please cleanse your mouth with the water cracker and rinse your mouth with water three times. Read all the instructions below before you begin

Place the entire sample in your mouth and make sure to start the timer provided.

Once the candy is in your mouth, start to vigorously tumble the sample in your mouth until the sample has completely dissolved. PLEASE AVOID CHEWING THE SAMPLE. You can swallow the saliva that accumulates. Stop the timer when you feel that there is no more candy left in your mouth.

SAMPLE: **456**

START TIME: _____ **END TIME: _____**

Did all of the candy dissolve in your mouth ? ☐ Yes ☐No (Go next question)

Which of the following was done to the remaining candy? ☐ Expectorated

☐Swallowed

Subject ID:_____

Durability Test

Black Raspberry Confections

CONFECTION # **890**

Please cleanse your mouth with the water cracker and rinse your mouth with water three times. Read all the instructions below before you begin

Place the entire sample in your mouth and make sure to start the timer provided.

Once the candy is in your mouth, start to vigorously tumble the sample in your mouth until the sample has completely dissolved. PLEASE AVOID CHEWING THE SAMPLE. You can swallow the saliva that accumulates. Stop the timer when you feel that there is no more candy left in your mouth.

SAMPLE: **890**

START TIME: _____ **END TIME:** _____

Did all of the candy dissolve in your mouth ? ☐ Yes ☐No (Go next question)

Which of the following was done to the remaining candy? ☐ Expectorated

☐Swallowed

Subject ID:_____

General Impressions

Black Raspberry Confections

To the best of your knowledge from your experience with the confections please complete the following statements.

1. I could consume two confections _____.

☐not at all ☐ four times per day ☐ twice per day ☐ daily ☐weekly

2. I could consume four confections everyday for _____.

☐not at all ☐ 3 days or less ☐1 week or more ☐ 1 month ☐ 2 months or more

What factors (taste, preparation, packaging, etc…) about the confections helped your ability to consume it daily?

_____

_____

What factors (taste, preparation, packaging, etc…) about the confections hindered your ability to consume it daily?

_____

_____

What changes need to be made to improve consumption of the confections?

_____

_____

What are your opinions towards consuming foods with added health benefits such as the black raspberry confections?

_____

_____

General Consumer

Black Raspberry Confections

1. Which statement best describes how you feel about **fruit**?
   ☐Like very much
   ☐Like moderately
   ☐Neither like or dislike
   ☐Dislike moderately
   ☐Dislike very much

2. How often do you consume **fruit**?
   ☐Never
   ☐Rarely (less than twice a week)
   ☐Occasionally (3 to 5 days/week)
   ☐Frequently (>7 days/ week)

3. How often do you consume **berries or foods containing them**?
   ☐Never
   ☐Rarely (less than twice a week)
   ☐Occasionally (3 to 5 days/week)
   ☐Frequently (>7 days/ week)

4. Which statement best describes your opinion about **black raspberries**?
   ☐Like very much
   ☐Like moderately
   ☐Neither like or dislike
   ☐Dislike moderately
   ☐Dislike very much

5. Which statement best describes your opinion about **candies with fruit**?
   ☐Like very much
   ☐Like moderately
   ☐Neither like or dislike
   ☐Dislike moderately
   ☐Dislike very much

6. How often do you consume **candies with fruit**?
   ☐Never
   ☐Rarely (less than twice a week)
   ☐Occasionally (3 to 5 days/week)
   ☐Frequently (>7 days/ week)

7. Which statement best describes your opinion about **candies**?
   ☐Like very much
   ☐Like moderately
   ☐Neither like or dislike
   ☐Dislike moderately
   ☐Dislike very much

8. How often do you consume **candies**?
   ☐Never
   ☐Rarely (less than twice a week)
   ☐Occasionally (3 to 5 days/week)
   ☐Frequently (>7 days/ week)

Appendix C – Health and Lifestyle Questionnaire

HEALTH & LIFESTYLE QUESTIONNAIRE

Date_____

Male ☐ Female ☐

Name_____ Birth date_____

Subject Information

Nationality / Race: _____ Occupation: _____

Home address: _____ Phone No. (H) _____

_____ (W) _____

Email: _____ (C) _____

Which is the best way to contact you email and/ or phone? _____ Best time to call? _____

Diet / Exercise

1. Do you have any allergies (food or medicines)? Yes ☐ No ☐
If yes, please specify your allergy and the extent of your allergic reaction:

_____

2. Have you ever eaten foods containing strawberries, corn, or wheat?
   Yes ☐ No ☐
3. In your regular diet, do you frequently consume red and purple colored fruits or vegetables? Yes ☐ No ☐
   If so, how many servings a week?
   <1 ☐ 1 to 3 ☐ 4 to 7 ☐ >8 ☐

4. Do you follow a particular diet? Yes ☐ No ☐
If yes, please check all that apply.

low fat diet☐	high fiber diet☐	low carbohydrate diet☐
no dairy (lactose free)☐	Atkins diet ☐	macrobiotic☐
The Zone diet☐	low sugar diet☐	40/30/30 diet☐
low sodium diet☐	_____(other)☐	high calorie diet (weight gain) ☐
diabetic diet ☐		low calorie diet (weight loss) ☐
vegetarian diet ☐		other_____ ☐

5. Do you exercise regularly?

If yes, please describe your weekly routine (please approximate time spent in each activity)

_____

_____

Medical History

For male participants please skip to question 4. The first three questions are for female participants.

1. Do you know or suspect that you are currently pregnant, are lactating, or plan to be pregnant during this 9-week study?

   Yes ☐ No ☐

2. Are you currently taking any oral contraceptives? Yes ☐ No ☐

3. Are you menopausal? Yes ☐ When was your last menses? _____
   No ☐ *Date*

4. Are you lactose intolerant? Yes ☐ No ☐
   If yes, how many servings of milk or dairy based products can you tolerate a day?0 servings ☐ 1serving ☐ 2 servings ☐

   Do you use lactase (Lactaid®)? Yes ☐ No ☐

5. Have you taken antibiotics in the last 6 months? Yes ☐ No ☐
   If yes, when_____ how long _____ reason _____

6. Have you ever had or currently have any medical problems with the following? If yes, check box

Blood (i.e. anemia, bleeding)	☐	Joints (i.e. arthritis)	☐
Chronic Illness (i.e. diabetes)	☐	Kidney	☐
Eating disorders (i.e. bulimia, anorexia)	☐	Large intestine	☐
Swallowing (dysphagia)	☐	Liver (i.e. hepatitis)	☐
Gall Bladder	☐	Small intestine	☐
Immune (i.e. lupus, cancer)	☐	Thyroid or Pituitary	☐

*If you have checked any of the above boxes, please describe the medical problem.

_____

_____

7. Have you ever had surgery on any of the following organs? If yes, check box

Stomach	☐	Intestines	☐	Thyroid	☐
Gall Bladder	☐	Liver	☐	Pituitary	☐

8. Do you take prescription medications? Please list with dose taken each day (if known).

_____ _____

_____ _____

_____ _____

_____ _____

9. Do you take vitamin or mineral supplements or dietary supplements? Please list with dose taken each day (if known).

_____ _____

_____ _____

_____ _____

_____ _____

10. Do you take any herbal, botanical or "alternative-medicine" preparations? Please list with dose taken each day (if known).

_____ _____

_____ _____

_____ _____

_____ _____

Dental/Oral History

Have you had any oral surgery in the last 3 months? Yes c Noc

If yes, where _____ When _____

Have you ever been told that you have periodontal disease (periodontitis)?

Yes c No c

If yes, when _____ Type/ severity ____

Did you need treatment for periodontitis?
Do you have any open sores or had any open sores in the last month? Yes c No c
If yes, where _____ when _____
Do you have any cavities or cracked teeth that have not been treated? Yes c No c

Are you currently using any prescribed or medicated mouth rinses? Yes c No c
If yes, name _____ reason_____
Do you brush your teeth daily? Yes c No c
If yes, how many times daily?___ _____

Do you floss your teeth daily? Yes c No c
If yes, how many times daily?___ _____

Smoking History
Do you currently smoke? Yes c No c
If **YES**, how many of the following: cigarettes per day? _____
*cigars per day?__*pipe use per day? _____
*Amount of tobacco can vary in these products therefore please provide as much detail about the size (small, medium, large cigar) or the amount of pipe tobacco used (1 teaspoon or tablespoon).

If **NO**, have you smoked in the past? Yes c No c (go to question 3)

If **YES**, you have smoked in the past
How long has it been since your last cigarette:
_____years_months

3-Day Diet Record

Subject ID: _____

Day 1: _____
Day 2: _____
Day 3: _____

3-DAY DIET RECORD

Instructions for Completing the Diet Record:

Try to eat the way you usually do and please keep in mind that you may be asked to further detail your diet recall at your next clinical appointment. Please follow the instructions below as carefully as you can. If you have questions, please ask the study coordinator.

Directions in completing your Three Day Diet Record

1. Record all the food you eat or drink for the day specified by the study coordinator. Most people find it helpful to note this as soon after the meal or snack as they can.
2. Write only one food item on a line.
3. Describe the **type of food** eaten as clearly as you can. Use the sample provided as a guide.
    - List ingredients to help describe any unusual casserole or salad.
    - Indicate whether the food is canned, fresh, frozen or diet.
    - List the brand names of foods if you know them.
4. Describe how the food was prepared.
    - Baked, broiled, fried, raw are examples
5. Remember to include all condiments such as pickles, catsup, tartar sauce, salad dressings, gravies and sauces.
6. Be sure to include any snacks such as gum or candy.
7. If you take a vitamin/mineral or other supplement consistently (at least once per week), please return the package label with your food records if it is available. Please list brand name, how many, how often you take them at the top of the first food record.
    - Ex: Centrum plus 1 tablet 5 days per week
8. Describe the **amounts** of food you eat and drink as clearly as you can. Use the following examples as a guide.

Practical guide to estimating portions

Thumb	1 ounce cheese
4 stacked dice	1 ounce cheese
Thumb tip to 1st joint	1 tsp
Bar of soap or deck cards	3 ounces meat
Palm of hand	3 ounces
1 ice cream scoop	½ cup
Fist or baseball	1 cup
Handful	1 or 2 ounces snack chips
Tennis ball	1 medium fruit serving
Computer mouse	½ to ¾ cup
Ping pong or golf ball	2 TBSP
Yo-yo or hockey puck	1 bagel serving

Specific information for estimating portions and recording

Liquids – list as *cups, parts of cups or fluid ounces

Meat, fish, cheese, eggs – list in ounces, by number, or size. Specify if the amount given is in cooked or raw weight. List bacon or sausage by number of slices or links.

Ex:	Chicken breast	Baked, boneless, skinless	3 oz.
	Lean ground beef patty	Broiled	¼ pound raw
	American cheese	Kraft singles	1 slice

Fruits – list as cups*, parts of cups, or by number. If possible, include the size (diameter and/or length) of fresh fruits

Ex:	Banana	1 small (6 inches long)
	Apple	1 medium (size of tennis ball)

Vegetables - list as cups*, parts of cups, or by number.

Ex:	Green beans	Del Monte canned	½ cup
	Baby carrots	fresh	10 carrots

Bread, rolls, crackers – list by number or size.

Ex:	whole wheat bread	Pepperidge Farm	1 slice
	Triscuits	Nabisco	4 crackers

Cereal, rice, noodles, potato - list by cups*, parts of cups, or by number.

Ex:	spaghetti	cooked, San Giorgio	1 cup
	Potato	baked	1 medium (about 4 inches long)

Pancakes, waffles – list by number and size

Ex:	pancakes	Betty Crocker, buttermilk	2 (5" diameter)

Jam, jelly, honey, syrup, sugar – list by teaspoons or tablespoons, one tablespoon is three teaspoons

Ex:	syrup	Aunt Jemima Lite	3 TBSP

Candy – list by number and size of bar (bite size, mini, regular or king) or pieces

Ex:	Sno Caps	3.1 ounce package
	Baby Ruth	1 regular size candy bar

Jello, puddings, ice cream – list as cups or parts of cups. Please indicate if pre-packaged or homemade.

Cookies – list by number and size

Ex:	cookies, Choc chip	Mrs. Fields	5 cookies (2 ½" diameter)

Pie, cake – list by number and size (length and width at the longest end).

Ex:	Ice cream cone, strawberry	Ben & Jerry's	½ cup
	Choc cake with chocolate icing	Homemade	1/10 of 9" layer Cake

Miscellaneous – list by teaspoons, tablespoons, parts of cups, or pats. Include butter, margarine, oil, sauces, dressings, gravies, dessert toppings added in cooking or at the table.

Eating Out
Give the name of the restaurant so that we may call for more info if necessary. Describe food items eaten as carefully as you can.

Ex: Pizza Hut Pizza Sausage and cheese 1 slice of medium 4" x 6"
McDonalds Quarter pounder with cheese 1 sandwich
French fries, McD's 1 small package about 30 fries

Example food record

Date/Time	Kind of Food	How Prepared or Brand Name	Amount or Size of Serving
9/17/2006			
7:30 AM	Cheerios	General Mills	1 cup
	2% milk	Kroger Brand	1/2 oz
	Banana		1/2 small
	One-A-Day vitamin	Women's formula	One pill
12:00 PM	Turkey sandwich		
	Bread	Home Pride, whole wheat	2 slices
	Turkey breast	Butterball	3 deli slices
	Reduced Fat Mayonnaise	Kraft	1 Tbsp
	Lettuce	Romaine	1 leaf
	Tomato	Fresh	2 slices
3:45 PM	String cheese	Mootown	1 oz
	Pepsi	Regular	12 oz (1 can)
	Corn Chips	Frito Lay	1 package (1.5 oz)
6:30 PM	Spaghetti	Mueller's	1 cup (cooked)
	Spaghetti sauce	Ragu, meat-flavored	3/4 cup
	String beans	DelMonte, canned	1/3 cup
	Lettuce	Iceberg, chopped	1 cup
	Tomato	Fresh	1/2 small
	French dressing	Kraft Fat Free	2 Tbsp
	2% milk	Kroger Brand	1 cup
8:30 PM	Ice cream	Breyer's strawberry	1/2 cup

Day I

Subject Number: _____ Date: _____

Day/Time	Food Item	How Prepared or Brand Name	Amount or Serving Size

Continued from previous page

Day/Time	Kind of Food	How Prepared or Brand Name	Amount or Serving Size

Day 2

Subject Number in study: _____ Date: _____

Day/Time	Food Item	How Prepared or Brand Name	Amount or Serving Size

Continued from previous page

Day/Time	Kind of Food	How Prepared or Brand Name	Amount or Serving Size

Day 3

Subject Number in study: _____ Date: _____

Day/Time	Food Item	How Prepared or Brand Name	Amount or Serving Size

Continued from previous page

Day/Time	Kind of Food	How Prepared or Brand Name	Amount or Serving Size